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Art. XVIII.—*The Endophytic Fungus of Lolium, Part I.*

BY

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(With Plates XVIII. to XXVI. and 8 Text Figures.)

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Historical Introduction.

The fact, that grains of *Lolium temulentum*, L. (Darnel) contain a layer of fungal hyphae, situated between the aleurone layer and the fruit and seed coat, was demonstrated by Vogl (1) in 1898. In the same year Guérin (2), Hanausek (3), and Nestler (4), published papers dealing with this subject.

These earlier workers drew attention to the fact that the presence of the fungus in the grain is a fairly constant feature. Guérin examined samples of *Lolium temulentum* from South America, Asia, Africa, and Europe, and recorded that only three showed the absence of hyphae. He failed to note their presence in the embryo, although they were observed in the ovary before the fertilisation of the ovum. *Lolium arvense*, With., and *Lolium linicolum*, Sond., were also found to be fungal-containing, but he reported only one example of *Lolium perenne*, L., with the fungus. He suggested that the presence of the fungus in the Darnel grains is probably an example of symbiosis rather than one of actual parasitism.

Hanausek's results confirmed those of Guérin, and, in addition, he noted the presence of the hpyhae in the nucellus of the young ovary, where, he stated, it produced knots. This fact, he suggested, indicates a possible affinity of the fungus in question with the *Ustilagineae*.

Hanausek never examined a Darnel grain without finding hyphae in the usual position, but all samples of *L. perenne*, L. examined showed the absence of hyphae.

Nestler working along the same lines traced the distribution of the fungus in the seedling, and in the growing plant right up to the formation of the grain. He, in addition, tried to cultivate the fungus in artificial media with negative results. Only a few grains were found to be devoid of the fungus. He examined several

of the species of *Lolium*, including *Lolium perenne*, *L.*, but found that the hyphae were absent in all cases. It was suggested that possibly Woronin's "Taumelroggen" and the fungus of Darnel bore some relation one to the other, on account of their somewhat similar physiological action. At the same time, however, he called attention to the many differences which might be cited between the two.

Hiltner's (5) attention was drawn to the work of Hanausek and Nestler, and in 1899 he published a paper dealing with the function of the fungus found associated with Darnel. This he stated to be of a nitrogen fixing nature, and proceeded to verify the statement by experiment. He recorded that *Lolium temulentum* grew equally well in nitrogen-free and nitrogen-containing sand, and he was thus drawn to the conclusion that the above statement as regards its function is the correct one. The methods employed by Hiltner are open to criticism, and I shall refer to his work in a later part of this paper (pp. 284-285.)

Micheletti (6), 1901, worked mainly on the chemical side of the question. A paper, "The Seed Fungus of *Lolium temulentum*, L., the Darnel," by Freeman (7), appeared in 1902. Freeman found that samples of Darnel from various localities showed wide differences in the proportion between fungal containing and fungal free seeds. He correlated the absence of the fungus with certain morphological characteristics, viz., colour and shape, although he indicated that in a few cases this correlation was not evident. Perhaps the chief point in his paper deals with the mode of entry of the fungus into the embryo. He described an isolated patch of hyphae at the base of the groove on the inner side of the grain. This patch he called the "infection layer" and he stated that it was from this layer that infection of the embryo took place. The course of the hyphae, according to his observations, was always intercellular, and penetration of the aleurone layer by the infecting hyphae took place at the junction of several cells. In all grains examined where hyphae were present in the embryo they were also found in the grain, and all the evidence was negative as to the possibility of their presence in the embryo and absence in the grain. However, he cited one doubtful case as regards this converse statement.

The distribution of the fungus in the growing plant was noted, and in dealing with the inflorescence and ovary he described in detail the development of his "infection layer."

All attempts to cultivate the fungus in artificial media were unsuccessful.

In conclusion he pointed out that Guérin considered the relation between the two organisms, one of true symbiosis; he agreed with this idea, but added, "the large hyphal layer of the grain, and the occasional penetrations of the endosperm, suggest vestigial indications that the action of the fungus is, or has been, at times injurious to the endosperm of the plant. Otherwise the fungus seems ordinarily to exert an almost stimulating influence on the host."

Freeman examined 30 grains of *Lolium perenne* L., and found only 5 contained the fungus. Of 59 grains of *Lolium italicum*, Braun, 2 alone showed the second organism, while of 25 grains of *Lolium linicolum*, Br., the full number gave positive results.

Another paper by Nestler (8) appeared in 1904, but it throws little further light on the problem. Fuchs (9), 1911, viewed the subject from the chemical standpoint, and finally, in 1912, a research by Buchet (10) was published, but, unfortunately, I have been unable to obtain this paper in Australia.

The erratic occurrence of the fungus in both *Lolium temulentum* and *Lolium perenne* recorded by these investigators does not tend to support the idea of a symbiotic association, but rather stresses the probability of its parasitic nature. The investigations described in the following paper were carried out in order to test these results for those grasses grown in Australia, and also to attempt to elucidate the actual relation between the two organisms. In attempting to further our knowledge of the relation between the grass and its associated fungus, I have limited myself mainly to a study of *Lolium perenne*, as practically no work has been done on this grass, and, in addition, it is a much more convenient form for obtaining embryological material. As far as time permitted I have compared this form with *Lolium temulentum*, and the results recorded in this paper are true for both forms. Perhaps a few minor differences may be determined later, but the main points are undoubtedly true for both grasses.

Methods.

Microtome sections were employed in the examination of the mature grains. The grains were soaked in distilled water for several hours, and then placed in a fixing fluid. During the early part of this work, Carnoy's fixing solution was used. Owing to the starchy nature of the endosperm, it was difficult to get good results,

but if a paraffin with a fairly low melting point be used, it was found quite possible to obtain good and serial sections, after using this fixing reagent. At a later stage Bouin's fixative was employed, more particularly when dealing with the later stages in the development of the grain. It was quite easy to obtain absolutely entire sections after the specimens have been fixed in this way. The disadvantage lies in the fact that the starch in the endosperm was not well preserved, and also after this fixative the staining reactions, with the stains employed, do not seem to be as brilliant as they are following upon Carnoy's fixative.

The ether-freezing microtome was not satisfactory, owing to the difficulty in obtaining serial sections, and it was generally necessary to do this. Again, it was impossible to obtain as thin sections in this manner as with the paraffin method.

Hand sections were practically useless. They can only confirm the presence of the hyphae in the grain, but evidence as to their absence cannot be drawn from them.

The stain most generally employed, in fact, solely, as regards the mature grain, was aniline gentian violet.[1] In using this stain care must be taken to see that it is always fresh, as it does not keep well. Its staining capacity diminishes rapidly after several days. This stain was washed out with Gram's iodine water, then with absolute alcohol. Sections were next cleared in clove oil, and mounted in balsam.

Excellent results were obtained with this stain, the hyphae for the most part being stained a brilliant bluish purple, and the endosperm reacting to the iodine.[2] It far exceeded any other stain I have tried, among them being Haidenhain's haematoxylin, aniline safranin, erythrosin, aniline blue, etc. The aleurone cells for the most part and the cells of the scutellum and embryo do not stain, so that the hyphae present in these tissues stand out in striking contrast to the colourless cells around them.

This stain, used by itself, was only useful when dealing with the mature grain. In studying the embryology of the grasses in question it was necessary to counter-stain. Sections of the ovary before, and at the time of fertilisation, were stained with Bismarck

1. One Soloid tabloid of gentian violet dissolved in 7 ccs. of abs. alc, and 63 ccs. of water containing 2.8 ccs. of aniline solution.

2. The colourisation of the endosperm by the iodine is, of course, not permanent, although the hyphae retain the violet stain well. This is certainly a drawback to the method, but it is more than compensated for by the excellent results obtained, which, indeed, are not approached by any other method.

brown, followed by aniline gentian violet; later stages were also treated in the same way. In addition, some of the later sections were stained with congo red. These were first stained with aniline gentian violet, followed by Gram's iodine water, and finally by congo red. This stain washes out very readily in the alcohols, so it was found necessary to use a watery solution of congo red, and to wash away excess with water, then to drain off as much of the water as possible, and transfer immediately to clove oil. If the sections be left on the water oven at a temperature of 45 -50°C., they will clear perfectly well in from 1-2 hrs., and they can then be mounted in balsam and prove to be quite permanent.

The mature grain.

My aim at first was to make a record of the grains of both *Lolium temulentum*, and *Lolium perenne* examined, and to note the number of fungus-containing and fungus-free seeds.

After examining a large number of grains, I have been forced to the conclusion that it is impossible to distinguish macroscopically grains containing the fungus from those devoid of it (if any). The colour difference cited by Freeman cannot be regarded as a distinguishing feature.

Nine grains were chosen from a sample of Darnel obtained from Northam, Western Australia. Of these 4 were very dark in colour, 2 more or less intermediate, and 3 a pale straw yellow, but all of the nine showed a dense hyphal layer situated between the aleurone layer and the outer testa and pericarp. This is but a single example of many similar series. As the work proceeded it became more and more evident that both colour and size of grain were quite independent of the fungal constituent.

When commencing this record hand sections were used, as it was possible to handle a large number of fruits in a comparatively short time, by this means. Sometimes these hand sections revealed a grain apparently fungus-free—i.e., no definite layer of hyphae could be seen in the usual position in the grain. These, when obtained, were frequently microtomed, and fine but distinct fungal hyphae were found penetrating the scutellum, so it seemed impossible to decide whether a particular grain was devoid of the fungus unless serial sections were obtained sufficiently thin to enable these fine threads to be demonstrated. Although hand sections are useful in demonstrating the presence of the fungus, they cannot be accepted as evidence in regard to its absence.

The following results show that *Lolium perenne* is just as striking an example of a fungus-containing fruit as *Lolium temulentum*, and that the number of either grains devoid of the fungus is remarkably small. In fact, they suggest that probably all grains of Darnel and English rye grass contain this second organism, and failure to discern it in some grains is due to the fact that it is present in such minute quantities in the mature grain that it needs special care and staining to bring the hyphae out, or, as this paper proceeds, a second alternative will be considered (p. 293).

Lolium temulentum, L.

Locality	No. of grains examined	Fung. pres.	Fung. abs.
Victoria	93	93	—
Northam, W. Aust.	9	9	—
Katanning, W. Aust.	27	27	—
Kew, England	31	31	—
Cambridge, England[3]	9	9	—
Total	169	169	

Lolium perenne, L.

Locality	No. of grains examined	Fung. pres.	Fung. abs.
Victoria	53	53	—
Cowra, N.S.W.	12	12	—
New Zealand	4	4	—
South Africa	18	18	—
Scotland	11	11	—
Ireland	17	17	—
Total	115	115	

Although former workers have recorded the presence of the fungus in *Lolium perenne*, previously it has been thought to be very sparingly distributed in this species. The above results show that this is not actually so. It has also been suggested that the toxicity of Darnel is due to its fungal component, but since English rye grass shows a regularly occurring hyphal layer as well as Darnel, this suggestion

3. The "seeds" of this Cambridge sample were much smaller than those of any of the Australian samples. Frequently also on hand-sectioning no hyphal layer was evident, but several of the grains were microtomed, and further examination then showed distinct hyphae in the scutellum and embryo. Possibly the plants yielding the grain were grown under conditions which did not favour the luxuriant development of the fungus, so that the absence of the extra-cellular hyphal layer was more common in this sample than is usually the case. Only these grains which were actually microtomed are included in the above list.

does not seem to be a feasible one. Of course it might be argued that the two grasses contain different species of fungi, one of which might be toxic, the other harmless. The actual identity of the fungi obtained from the grains can only be established when they are grown artificially, and the sporing stage obtained, but as far as one can judge by comparing the two forms, they are very similar, and are certainly very closely related if not actually identical. Seemingly the explanation of the cause of the poisonous nature of Darnel must be looked for elsewhere, and is not to be furthered by a study of the fungus found inhabiting it.

Freeman, when discussing the fungus in the embryo of the grain, records some experiments which were undertaken in order to investigate the function of the nucellar layer of hyphae, although it is not quite clear what bearing they have on this point. He grafted embryos of *Lolium temulentum* on endosperms of *Lolium perenne*, and vice versa, the grains having previously been sterilised, and all manipulations carried out under sterile conditions. Thirty-four grafts of *Lolium perenne* embryos on *Lolium temulentum* endosperms were made, and of this number eighteen germinated. He examined two of these seedlings, and found both contained hyphae, from this he argued it was very probable that "hyphae from the *infection layer* of the *L. temulentum* grains were able to gain entrance to the embryos of *Lolium perenne*." These experiments really lead nowhere, for the hyphae are already in the rye grass embryos before grafting on any foreign endosperm, and their presence cannot possibly be due to infection from the nucellar hyphal layer or from his localised *infection area*.

Distribution of the fungus in the grain.[4]

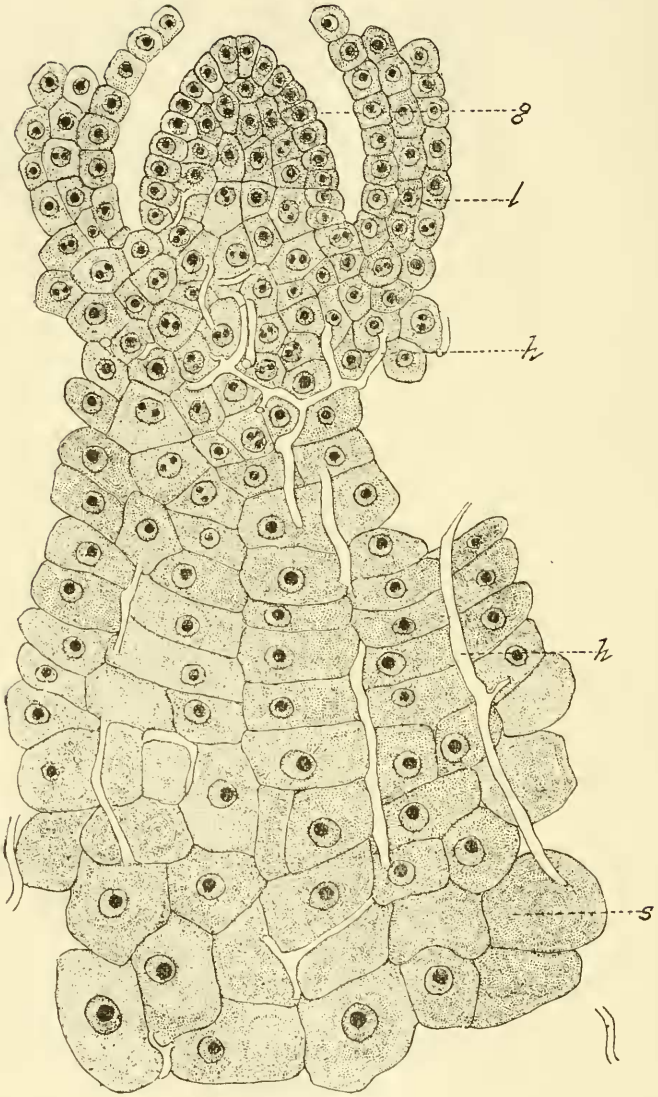
Many grains of *Lolium perenne* were sectioned with a view to determining the distribution of the fungus in the fruit. Transverse sections taken at different levels are shown in Plate XVIII., Figs. 1-3. The yellow line illustrates the distribution of the fungus. A transverse section at the distal end of the grain, Fig. 1, shows the hyphal distribution to be co-extensive with the aleurone layer. A

4. Following upon Brown and Morris (11), I have adopted the following terminology in describing the grain. The furrowed side of the grain is the *ventral* surface, the side opposite to this the *dorsal*. The embryonic end is called *proximal*, while the stigmatic end, or that portion remote from the embryo, is *distal*. A section passing through the ventral and dorsal surface is a *sagittal section*, while the longitudinal section at right angles to this is a *coronal* section. The sagittal plane which divides the grain into two equal halves is the *median sagittal plane*. A section at right angles to both sagittal and coronal planes is *transverse*.

transverse section nearer the proximal end of the grain, as is shown in Fig. 2, would cut through the scutellum. Such a section shows that the hyphae occur between the endosperm and the scutellum wherever the tissues are in contact, and hyphal penetrations into the scutellum may and do take place at any point over this area. The hyphae in addition extend even past the limits of the aleurone layer, and penetrate the scutellum on its outer exposed dorsal surface. A transverse section at the extreme proximal end of the grain, Fig. 3, passes through the embryo, but the starchy endosperm is no longer included in the section. Even at this level the hyphae surround the scutellum, as is indicated by the yellow line in the figure. The coronal plane is perhaps the best for demonstrating the distribution of the fungus in any one section (Plate XVIII. Fig. 4), the occurrence of the fungal layer between the scutellum and endosperm at all points of contact and the extension of the fungal tissue on the dorsal proximal surface is clearly seen.

Plate XVIII. Fig. 5, shows a median sagittal section illustrating the same points and, in addition both of these latter sections shew the distribution of the hyphae in the embryonic area. The scutellum is often very richly traversed by fine fungal threads, and they are not restricted to any special area, but occur more or less uniformly right through the tissue. Some grains show these threads more readily than others, but a study of the embryology of the grain will suggest that this might often be the case. The hyphae are readily discernible in the growing cone; their presence here has been pointed out by the earlier workers. (Text-figure 1.). The above facts are also true of *Lolium temulentum*, but it is much rarer in this case to obtain a scutellum so markedly inhabited as in *Lolium perenne*, and in any case the threads are generally finer. In several examples of *Lolium perenne* I have found hyphae present in the radicle, but they are not generally evident in this region.

Freeman raises the question—How does the fungus obtain entrance to the embryo? As an answer, he devoted a large part of his paper to a description of a localised patch of hyphae, which he termed "*the infection layer*," and to its mode of origin. He says that on the ventral proximal end of the grain there occurs an isolated patch of hyphae which penetrates between the aleurone cells and cells of the scutellum, and thereby gain entrance to the embryo when it is fairly advanced in its development. He states that on the dorsal surface of the grain the hyphae do not extend to the end of the aleurone layer. To



Text-Figure 1.

Growing point of an embryo from a *Lolium perenne* grain. The section was cut obliquely and includes only the growing point =g; young leaves=l; and scutellum=s; hyphae=h. × 850 diam.

quote directly: "It is not impossible perhaps that infection may, in exceptional cases, take place from this side of the scutellum (dorsal); but, if so, it occurs very seldom. I have seen no evidence either in the mature grain or in the developing ovary to indicate that such an infection is ever accomplished."

My observations permit of a different answer to this question. Hyphae occur at the junction of the scutellum and endosperm, not only near the ventral surface (Freeman's infection layer), but wherever these tissues are in contact I was unable to demonstrate the existence of an isolated patch as described by Freeman. Furthermore, it is impossible to agree with the statement that the hyphal layer does not reach the end of the aleurone layer on the dorsal side of the grain. As is shown in Plate XVIII. Figs. 2-5, hyphae can and do occur right round the periphery of the embryonic area.

These facts in themselves are interesting, but they do not answer our question. At a later stage, in this paper, it will be shown that *infection* of the embryo takes place at a very early stage in development, and that the distribution of hyphae in the mature grain has no bearing on this point, but is a result of the special function carried out by this partner in the development of the grain.

It is only fair to emphasise the fact that Freeman dealt only with *Lolium temulentum* when working out his idea of an infection layer, and that this criticism is based *mainly* on work done on *Lolium perenne*. However, if the facts demonstrated in the embryological section (pp. 267-281) are true, they apply equally well to both forms, and it becomes abundantly clear that the distribution in the adult grain is not associated especially with the *infection* of the embryo as Freeman suggests.

Previous workers have described the hyphal layer itself in detail. Australian grown grains of either grass seem to shew a very rich growth of hyphal tissue. Some grains of Darnel grown in the University grounds, Melbourne, had an average layer of 31.6 u. Grains of English rye in many cases showed a layer quite as broad as that shown by an average Darnel, but in both the width or extent of the layer is extremely variable, depending largely on the activity of the fungus during the period between fertilisation and formation of the seed. Aniline gentian violet, followed by Gram's iodine water, was used solely for staining the adult grains. The hyphal layer does not stain uniformly, however, with this stain, some portions of the hyphal threads reacting to the violet colour, other parts remaining colourless. This variation in the staining

properties was displayed by different parts of the same hyphae, the coloured portions being interrupted by colourless, in a very irregular manner. In order to ascertain whether these unstained segments contained protoplasm or were devoid of contents, and thus remained unaltered by the stain, sections were submitted to a second stain following upon gentian violet. Congo red was chosen, as it stains the cell walls, and also the protoplasm The result was that the former uncoloured sections were stained with the red, and displayed dense contents just as is the case with the coloured segments The difference in the staining capacity is probably due to the presence of ferments in certain parts of the hyphal network wherever the ferment is present in any quantity, then will the "blue" stain be evident. Colour is lent to this idea by the fact that the aleurone layer shows the same staining reactions as the hyphal layer. The majority of the cells do not react to the violet stain, but certain of them stand out markedly from the rest, for they stain densely and form very striking portions of the section. The number of such coloured cells varies in each individual grain. In addition, the scutellum repeats the above phenomenon. In this case, the "blue" cells are generally restricted to the epithelial layer of this tissue.

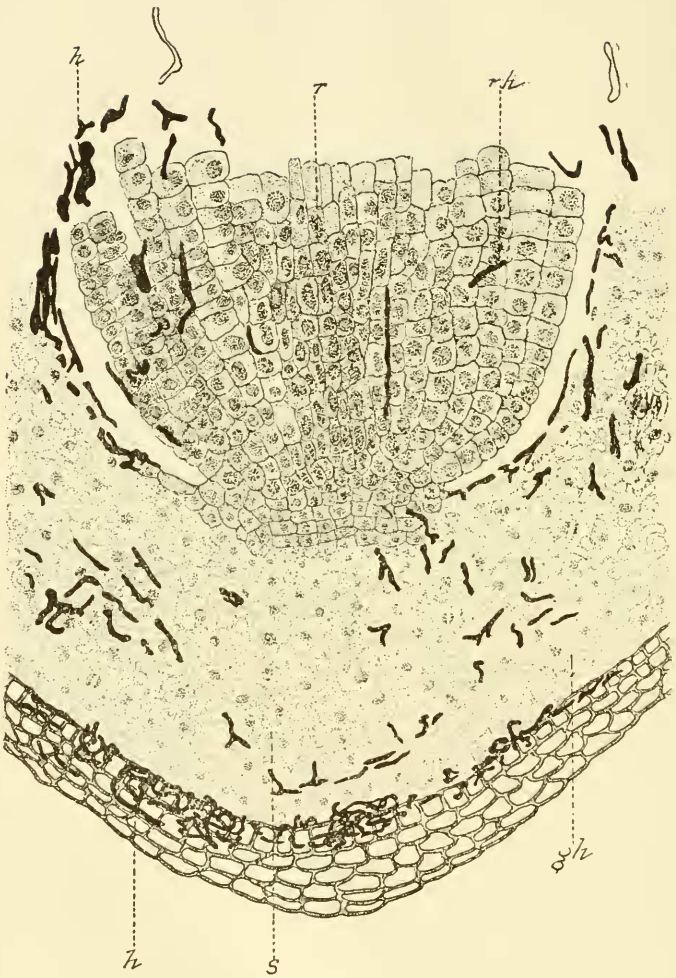
Brown and Morris (11) have shown that in *Hordeum vulgare* the secretion of diastase is located in the absorptive epithelium, and in a later paper Brown and Escombe (12) that in addition, the aleurone layer is capable of bringing about marked changes in the endosperm when this is separated from its embryo, and placed under favourable conditions.

The distribution of the active ferment-secreting cells therefore, agrees with the staining reactions described above, and supports the view that the coloured segments of the hyphae contain either an enzyme or its fore-runner, and this conjecture is further strengthened by the later embryological work.

When examining a sample of English rye grass from Ireland, a specimen was occasionally found showing hyphae (which for the most part stained with gentian violet) invading the starchy endosperm. Freeman records a similar distribution for grains of *Lolium temulentum* from Ghent. A careful examination of the aleurone layer of such a grain showed that the hyphae were also running riot here. Instead of the usual inter-cellular course, many hyphae could be made out actually passing into the cells, and in many cases a single hypha could be traced entering and leaving

as many as three cells. As is well known, the cell walls of this layer are thick, and also pitted. The hyphae enter through these pits, and thereby gain access to the cell (Plate XIX. Fig 5). Sometimes the opening in the wall of the aleurone cell was smaller in diameter than the penetrating hypha; when this was the case a conspicuous narrowing was noticed at the point of entrance, but on the far side of the pit the hypha again attained its previous size. In addition the scutellum showed an extraordinarily large amount of the fungus. Here the intra-cellular course was also very evident (Plate XIX. Fig. 4). Many of the scutellar cells stained vividly; such cells were seen to be fungal containing. The entrance to the cells was gained through pitted walls, as is the case in the aleurone layer. The remaining cells of the scutellum were normal, and the grains did not seem to be any the worse for this exceptional behaviour on the part of the fungus. In such abnormal grains the hyphal layer was present as usual. There is no doubt that the hyphae invading the cells are the same as those composing the extra cellular layer.

These phenomena were not confined to the sample from Ireland, one of English rye grass from South Africa also contained certain grains showing an extraordinary distribution and growth of the fungus. As before, both the aleurone layer and the scutellum were permeated by intra-cellular hyphae. In one particular case the scutellum, which normally is packed with aleurone grains, appeared to consist of a dense sclerotial-like mass of threads. The bulk remained colourless, and they resembled "ghosts," or casts, of former more virile hyphae (Text figure 2). They are represented in the text figure as dotted lines, and they completely filled the whole of the scutellar tissue, although the cells composing it were not distorted or enlarged in any way. This section cut in the coronal plane) and the others accompanying it, were later stained with congo red; it was then easier to decipher these ghost-like contents of the scutellum. Many were cut transversely, but owing to a large amount of twisting some were seen running lengthwise through the tissue for a short distance. They probably represent fungal hyphae, which were numerous at certain stages in the development of the grain, carrying a special food supply to special parts, and in giving this up to the host-plant they have undergone a partial dissolution, which was not completely carried out in these few exceptional cases by the time the grain reached maturity.



Text-Figure 2.

A longitudinal section through the radicle, and a portion of the scutellum of a grain of *Lolium perenne* (Ireland). This was an abnormal grain. r = radicle, rh = hyphae in radicle, s = Scutellum, gh = ghost-like hyphae forming a sclerotial like growth in the scutellum which was nevertheless perfectly formed; h = hyphae staining with gentian violet. × 103 diam.

Text-figure 3 illustrates the stigmatic end of the same grain (a coronal section not cut in the median line shows portion of the

Text-Figure 3.

A section of the stigmatic end of the same grain as text fig. 2. a = aleurone cells, the outlines of which are distorted by abnormally large intercellular hyphae; h = intercellular hyphae, h = intra-cellular hyphae, w = wall of aleurone cell. × 1000 diam.

aleurone layer at this end of the grain cut tangentially, and therefore it does not appear as a single layer of cells.) Interpolated between the aleurone cells, lying in the inter-cellular spaces, altering their whole contour, are outlines of hyphae, which seem to be swollen, somewhat gelatinised, and in a state of disorganisation. Similar bodies were also visible in the matrix of the cells themselves.

These occurrences lead me to believe that at some stage in the life of the grain the hyphae were intra-cellular, and that in the few aberrant cases met with this embryological condition persisted in the mature grain.

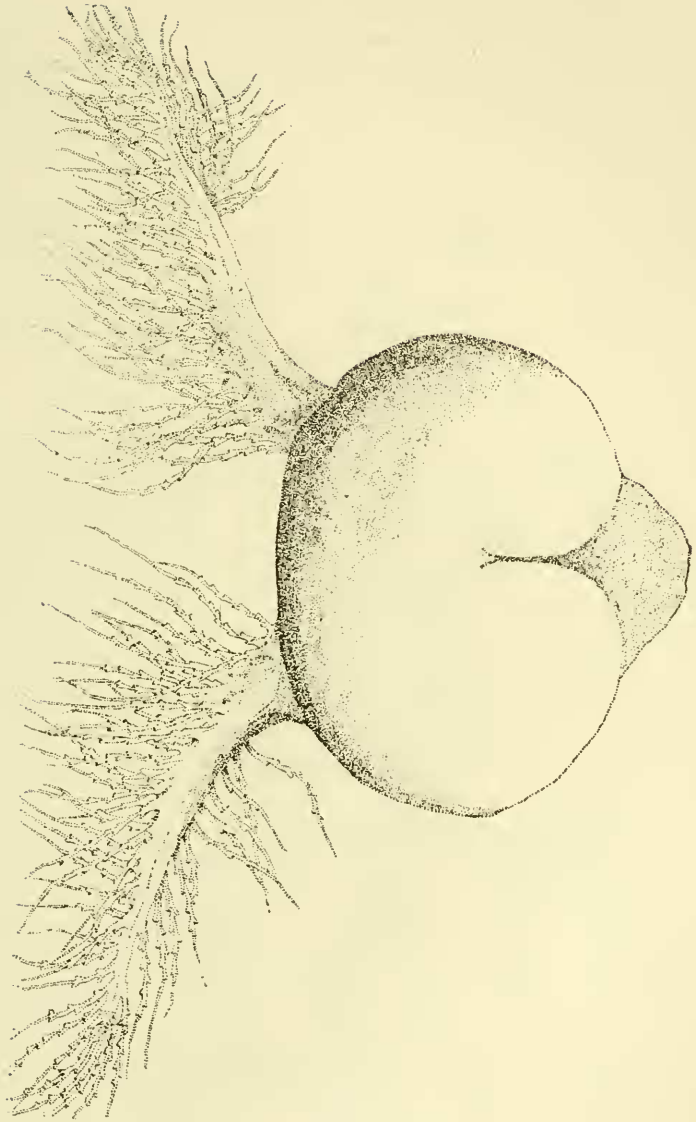
Development of the Ovary from the Flowering to the Fruiting Stage.

It is convenient to divide this portion of the paper into sections, and to consider the relation of the fungus to the grain, at certain definite stages in its formation. This relation becomes very pronounced and characteristic, either just about the fertilisation period or immediately afterwards, and from here to the final stages is most intimately associated with the changes taking place, resulting in the formation of the endosperm, with its aleurone layer, and the various parts of the embryo.

Stage A.

Text-figure 4 illustrates the external appearance of the ovary at the flowering stage just prior to fertilisation. It is drawn from the ventral surface, and shows the stigmas arising from the dorsal side, the bi-carpellary nature of the fruit is indicated in the figure. The ovum lies directed towards the proximal end of the ovary. I have designated this period Stage A.

Hyphae are present in the carpels from their earliest inception, but it is only at about this stage that their intimate relation with the ovarian tissues of the grass is evident. They enter the ovary at the stalk end, and branch through the carpellary wall. They are generally more abundant during the earlier stages at this end than at the distal stigmatic end. These hyphae characteristically accompany the vascular tissue of the stalk, and are to be seen in very close proximity to the annular and spiral vessels running in this area. (Plate XXI. Fig. 4.) In many of the sections numerous small lateral buds on the hyphae suggested haustoria, but they may be minute lateral branches just being caught in the section. The



Text-Figure 4.

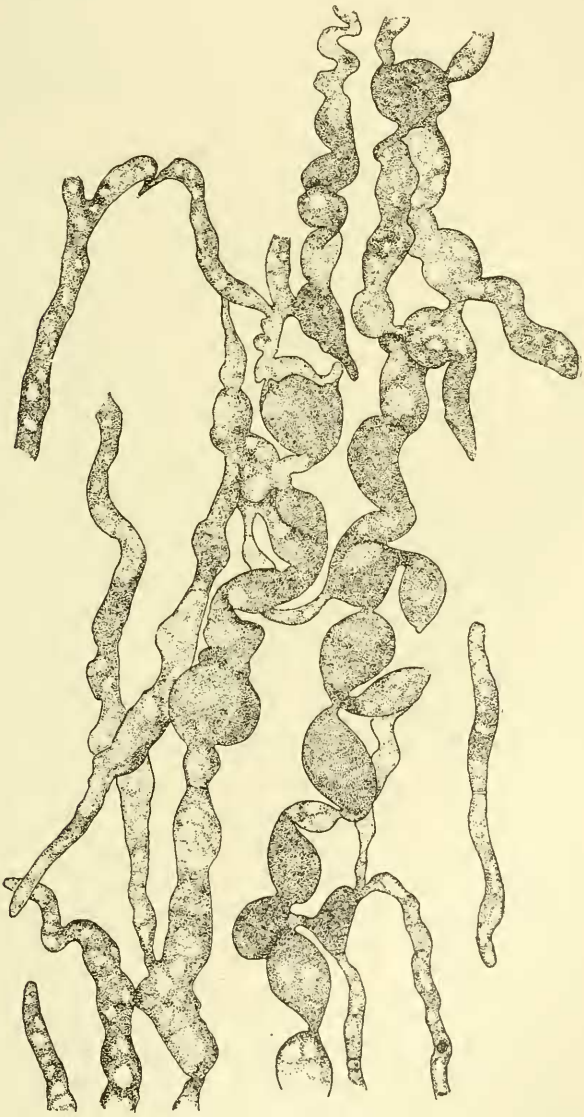
External appearance of the ovary of *Lolium perenne* at Stage A. × 15 diam.

cells of the carpellary stalk do not contain starch, and many of them stained deeply with the gentian violet after counter-staining the sections with bismarck brown. These blue cells were always plentiful in this region; such cells have been invaded by the fungus, and their contents probably used for its own nutrition. They always stain deeply, and the hyphae in their vicinity do likewise. If the cells do not stain too darkly, it is possible to observe fungal threads forming a network in the lumen of the cells, many of the threads being exceedingly fine. The position of these cells is shown in Plate XX. The cells are not enlarged, and apparently only differ from those around them by their different staining properties. The cell walls in this area are pitted; whether this is the normal condition or whether the pit has resulted from a secretion of the fungus is a debatable point.

The lateral walls of the carpel are packed with small compound starch grains, and in this region the hyphae only occur between the cells. They run in all directions, but are, as far as I have observed, strictly inter-cellular in this position, at this stage. However, when the stigmatic region is reached they seem to get the upper hand, and a large number of cells become their prey. These cells are also starch-containing, and when so intruded upon they immediately react to the violet stain. Sometimes the whole of this area will appear a dense violet colour, for the great majority of the cells in this part are attacked at this period. When the cell is first invaded, the starch is seen to become swollen and disorganised, and loses its power of reacting to the iodine wash used in preparing the sections (Plate XXI., Figs. 2 and 5). The fine hyphal threads wrap round the starch groups, and even enter between each individual grain (Plate XXI. Fig. 5), apparently digesting them. There is no doubt that these cells are suffering at the hands of the fungus, and that their contents are being transferred to this fungal system. Some of the cells show an entire absence of starch; they appear to be practically empty, and somewhat collapsed. These have been invaded at an earlier stage, and yielded their contents in a similar way. The stigmatic tracts present in the carpel wall generally show hyphae in abundance; they extend right into the stigmas, and even here become intra-cellular, but do so probably only after fertilisation has taken place, when the function of the stigmas has been completed.

Occasionally, the base of a staminal filament remained attached to the ovary during sectioning, and hyphae were found to extend

into this region. One or two examples were obtained, showing such hyphae in a much convoluted condition. Parts of the thread were swollen and bladder-like, with sharp constrictions at intervals. The contents, however, were the same throughout the length of the thread, showing no signs of spore formation. (Text-figure 5.)



Text-Figure 5.

Hyphae from a staminal filament shewing sharp constrictions occurring at intervals along their length. × 1700 diam.

While these changes are proceeding in the carpel wall, the hyphae in the developing ovule are not quiescent. They keep pace with the growth of the ovule, and until the embryo-sac is at the 8-celled stage they simply run between the cells of the nucellus, ramifying in every direction. They extend right through the nucellar tissue completely surrounding the embryo-sac. Freeman, when discussing the ovary of *Lolium temulentum* at this stage, states that hyphae are completely wanting on the outer dorsal surface towards the embryo-sac end, stopping at about the level of the antipodal group. If this is so, it is difficult to see how hyphae come to be present in this position in the mature grain. As far as I have observed they are uniformly distributed through the inner layers of the nucellus, but do not generally extend into the very outer layers until later in development. The dual staining properties are shown by these hyphae, but the great majority of them will pick up the purple stain.

The first indication of any change in the relation between the fungus and the cells of the ovule at this stage is the tendency for the hyphae to form knots (Plate XXI. Fig. 3). These are especially striking if the sections are cut rather thicker than those to be used for detailed high power examination. Hanausek described the occurrence of knots (Knäuel) in the ovary of Darnel, and figured them. I have been unable to obtain his original paper, only abstracts without figures being available. He offered the occurrence of these knots as evidence in favour of the fungus being related to the *Ustilagineae*. Freeman says: "I have found no such knotting of hyphae to indicate the commencement of Ustilagine spore formation." These knots undoubtedly do occur, but are rather to be regarded as the first stages in the penetration of the nucellus cells. The hyphae arch round all sides of the cell before entering it, and as they generally invade two or three adjacent cells simultaneously, this arching gives the knot-like formations above described. I do not think they afford any clue to the actual systematic position of the fungus in question. Since they are just on the point of attacking a cell they are rich in ferments and always stain vividly.

Cells showing a later stage of invasion are also present in such an ovule. Lateral branches arise from these enfolding hyphae, which penetrate the cell wall and pass into the substance of the cell itself. It soon becomes filled with a dense network of threads, and in this condition forms a most striking part of the

section, for such cells are the only members of all the nucellar tissue, which will stain in the same way as the fungal system. (Plate XXI. Fig. 1). They stand out in contrast to the background of normal, unattacked nucellar cells.

It is difficult to determine whether the hyphae actually apply themselves to the nuclei, but it is readily seen that the nuclei do undergo a definite change, becoming large and somewhat distorted, and at this stage will stain uniformly with the violet dye. These fungal-containing cells may occur in any position in the nucellus, but at this stage they are few in number, and are generally to be found at the end of the ovule furthest from the micropyle. They become more abundant after fertilisation occurring in any part of the nucellar tissue.

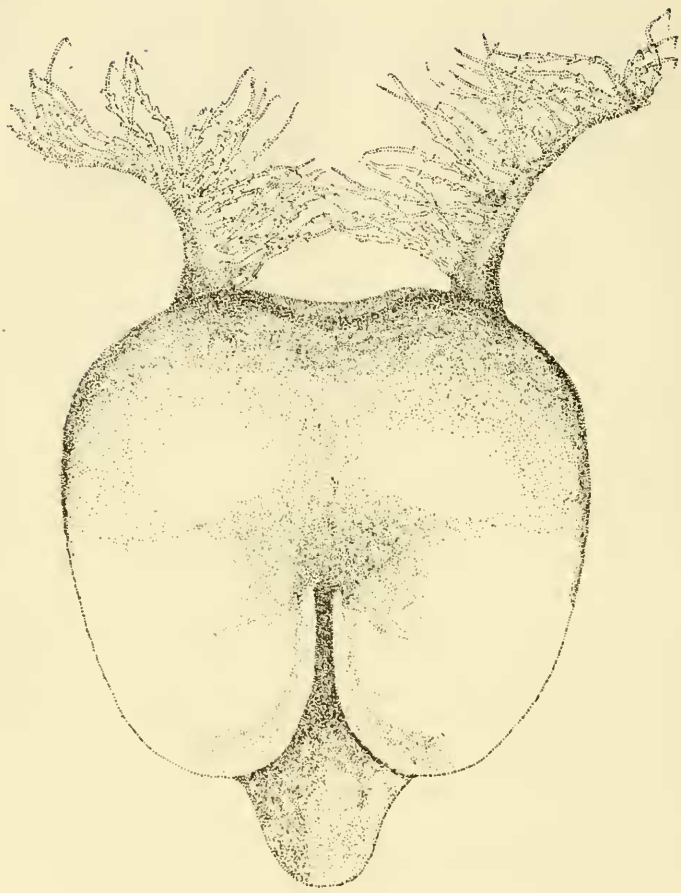
The embryo-sac at this stage is ready for fertilisation, and it agrees with the rest of the ovule in containing the fungus. The protoplasmic lining of the sac carries the hyphae. They run rather sparingly along the sides, and at the distal end of the embryo-sac, but are more abundant at the proximal end in the vicinity of the ovum. They are in close connection with both the synergidae and the egg-cell, and enter into the substance of the latter at this early point in the development of the grain. (Plate XXI., Fig. 6.) Previously it has been thought that "*infection*" took place at a much later stage, after the differentiation of the growing cone, when the formation of the embryo was fairly advanced. It has been suggested that the entrance of the fungus into the embryo was due to the chemotactic influence of the growing apex. My observations show that the fungal constituent is present in the ovum before any divisions have taken place, and that the formation of a special layer in the grain for the purpose of infecting the embryo at any specified period is not necessary.

Stage B.

Text-figure 6 illustrates the external appearance of the ovary after fertilisation, and at the commencement of endosperm formation. The elongation of the ovary which accompanies this change is beginning to be apparent. As in Stage A, it is drawn from the ventral surface, and shows the same features as before.

If an ovary be sectioned at approximately this stage, our knowledge of the relation of the fungus to the grass is considerably augmented.

The hyphae are still active in the carpel wall. The cells composing the distal area of this wall are attacked by the fungus, their



Text-Figure 6.

The external appearance of the ovary of *Lolium perenne* at Stage B. × 15 diam.

starchy contents are disorganised and absorbed into the hyphal system. The collapse of these cells enables the ovule to encroach on the space formerly occupied by them, and elongation of the developing grain thereby results. At this period the ovule does not increase markedly in breadth, and so the cells composing the side walls of the carpel are not yet invaded, the activity of the fungus is, as in Stage A, more evident in the distal region.

The most noticeable change occurs in the ovule itself. The fungus is responsible for the disorganisation of the nucellus.

Thriving on the nutriment obtained from these cells, and also on that obtained from the carpellary wall, it increases tremendously in amount, invading and attacking every portion of this tissue. (Plate XXII. Fig. 1.) This figure represents the hyphae massed together in this area. For the most part it is difficult to discern the outlines of the disorganising cells, except at the edge of the ovule, where they are still intact, although, unlike the previous stage, the hyphae have spread now into these outer layers. The nuclei of the cells of the nucellus persist for some little time after invasion, but they become enlarged, and stain uniformly, as shown in the figure.

The type of branching of the hyphae is very characteristic. (Plate XXII. Fig. 2.) The branches are given off almost at right angles to the main thread, and at their point of origin a slight swelling generally occurs. They are strongly septate, and rich in protoplasmic contents, and they show numerous vacuoles and well marked nuclei. If the sections are stained only with gentian violet, followed by Gram's iodine solution, the majority of the hyphae in these regions stain deeply, but the colourless portions noticed both in the adult grain and in the ovary prior to fertilisation are still present. In order to stain these segments sections at this stage were subjected to congo red, after staining in the above manner. Such treatment made the study of the endosperm much simpler.

The embryo-sac as a result of the stimulus of fertilisation has enlarged considerably, the enlargement being accompanied by the appearance of endosperm. The formation of this tissue is at first most active at the proximal end of the sac, in the vicinity of the ovum. On the dorsal proximal surface it forms a complete plate of tissue, the distal extremities of which are separate and considerably narrowed. These dip towards the ventral surface, and in section appear as two bands of tissue, from one to two cells in width, each being surrounded by nucellus.

The cells of which the endosperm is composed are highly protoplasmic, and contain large nuclei. Starch has not, as yet, been laid down in them. The endosperm is formed at first by a process of free cell formation. This soon ceases, and further growth takes place by the repeated division of the outer layer of cells, and thus the tissue grows, and gradually assumes its mature condition. This mode of growth is more easily followed at a later stage, so further reference will be made to it when dealing with Stage C.

Until the endosperm commences to be formed, the fungus has been increasing in amount at the expense of the nucellus, etc. This increase is only a temporary one, for the hyphae now grow in close contact with the endosperm cells. They enter them when the cells are young and not fully formed, and are here seen to become disorganised. The food material thus gained by the grass is used in the preparation of the reserve store of food, which is later to be deposited in this tissue. Plate XXII., Fig 3, shows a portion of the endosperm and the accompanying hyphae. This section was stained with congo red, and the hyphae and protoplasm stain in the same way. Plate XXII. Fig. 4, also shows the close union between the fungus and the grass. This section was stained only with gentian violet, and the hyphae could be traced more readily in the cell itself. Many of the disorganising threads running in the host cells stained blue, and are shown in the figure, the cells themselves remaining unstained. Plate XXII., Fig. 5, repeats the structure shown in the two previous figures, but in addition it shows extremely well, lateral branches, which arise from a hypha running parallel to the length of the endosperm, and which enter adjacent cells of this tissue, yielding up their food to the embryo grass plant.

The fungus is most abundant in the region of the ovum, due probably to the fact that the lumen of the embryo-sac begins to fill first around the embryo. In this region the cells are long and crescent shaped, and have very dense contents.

The synergidae are still present, and their absorption is no doubt the result of the activity of the fungus, a fact which may help to explain the pronounced growth of hyphae always present in this position.

The ovum is still undivided, although it has increased in size and the cytoplasm has become vacuolar.

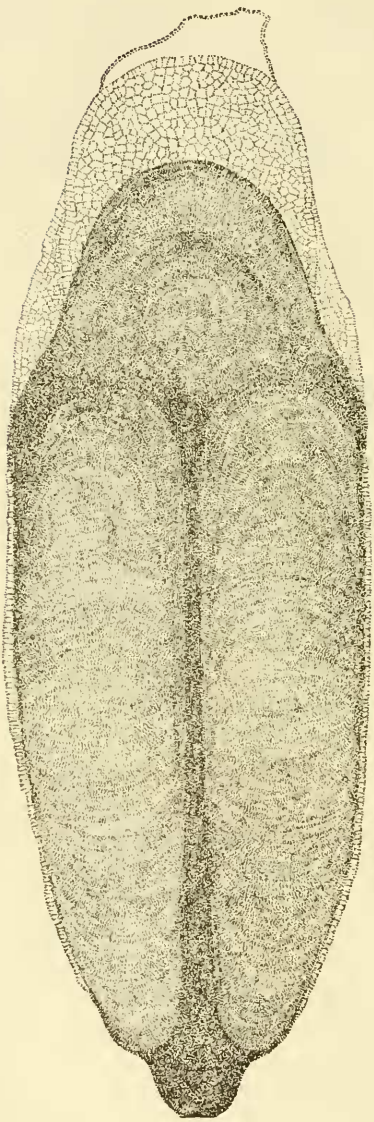
In the intermediate stages between B and C, the division of the ovum and the subsequent growth of the embryo are points of interest. The first division of the egg is generally transverse, at right angles to the pro-embryo, and each cell usually contains a well-marked vacuole. The fungus ramifying in the nucellus in this part of the sac comes into direct contact with the endosperm, which forms a lining to the pocket in which the embryo grows. The hyphae are unusually abundant, and are actively transferring food-material from the various parts of the carpel to the endosperm in this area. (Plate XXV., Fig. 1.) These cells are later absorbed

by the growing embryo, which, therefore, ultimately benefits by this concentration of hyphae. The cells of the pro-embryo also receive hyphae directly. Plate XXV., Fig. 3, shows a more advanced embryo. The dermatogen has just been cut off, and the hyphae are seen to enter right into the substance of the embryo. The endosperm cells at the extreme micropylar end are disorganising as a result of the presence of the fungus, and are also included in the figure.

Stage C.

Text-figure 7 illustrates the external appearance of the ovary when it is approaching maturity. A considerable amount of endosperm has been formed; the shadowed portion of the diagram indicates the extent and distribution of this tissue. As in the two previous stages, the ovary is drawn from the ventral surface. The furrow is noticeable at the proximal end, but as yet, is not well developed at the distal extremity. The dorsal proximal end projects beyond the rest of the ovary in the form of a pocket, in which the embryo develops.

Sections taken at this stage emphasise the facts already disclosed. The great bulk of the nucellus has disappeared, whilst the endosperm has increased in a well-marked and definite manner. The hyphae are still abundant, but owing to their absorption by the endosperm they are not as plentiful as in Stage B. The disorganisation which takes place all round the periphery of this layer keeps the growth of the hyphae in check, and, consequently, they never over-run the developing grain, but tend to decrease in amount after the first appearance of endosperm. (Plate XXIII.) The food-supply made available to the grass by the digestion of the hyphae is utilised by the young actively growing endosperm cells. This is rendered possible, for the growth in size of this tissue takes place from the outer surface. The outermost layer of the endosperm may be regarded as a cambium, which is active only on its inner surface. This meristematic layer divides in the usual manner, and the cells so formed are at first more or less brick-shaped, but gradually assume an approximately spherical form, and attain their adult size. Growth is carried on in this manner until the fruit is practically mature, then this outermost dividing layer ceases its activity, but persists in the grain as the aleurone layer, the cells of which serve as a store of nitrogenous material. This idea of an *endospermic cambium* is supported by the fact that the nuclei of this layer remain large and intact, even when the cells are



Text-Figure 7.

The external appearance of the ovary of *Lolium perenne* at Stage C. × 15 diam.

packed with reserved food, and also the walls of the aleurone cells become thickened, showing pits or points of communication at intervals. It is a well-known fact that cambial cells embarking on a period of rest show considerably thickened cell walls. The thickenings are usually removed wholly or in part, when such a layer recommences its activities.

If the stamens, before they ripen, are removed from a spikelet and cross-pollination is also prevented, the ovary remains small, but the fungus will grow rapidly in the nucellus, and ultimately forms a sclerotial-like mass of hyphae, occupying the main part of the ovule. Freeman records this fact for Darnel, and I have had similar results with English rye grass. Since, by preventing pollination, endosperm formation does not take place, this dense growth is only to be expected, for even in a fertilised ovary the fungus is parasitic on the nucellus, and for a short period tends to increase in amount. The prohibiting factor is the endosperm, which destroys the hyphae as fast, or faster, than they are being formed, hence removal or absence of this factor favours the development of the fungal organism, and the attempt towards sclerote formation is the result.

The primary tissues have been cut off in the embryo. It has elongated considerably in length at the expense of the endosperm cells adjacent to it. (Plate XXV., Fig. 3). These no longer form a close investment to the embryo, but have disappeared at the micropylar end, and the embryo now lies free in the embryo-sac. The attachment of the suspensor to the micropyle is broken at this stage. Hyphae still run in close association with the embryo, and in section it shows the hyphae running in its tissues.

Transition from Stage C to the Mature Grain.

Externally, the only changes which are evident during this transition are the elongation of the ovary, accompanied by an increase in breadth, and the development of the embryonic area, which becomes more pronounced as the scutellum develops and the embryo reaches maturity.

Sections taken at any stage during this period show features common to the earlier stages. The increase of endosperm, resulting from the continued activity of its outer layer, tends to crush the hyphae, which are ramifying in the remnants of the nucellus, into a layer running round the periphery of the seed. This layer becomes more pronounced as the endosperm reaches its adult size, and fills

the space formerly occupied by the nucellus, the outer parts of which are the last to disappear. Starch cells continue to be formed until the seed is almost ripe, and the hyphae nourish these young starch cells, just as they did the inner, now mature, starch cells, in the younger stages already described.

When the endosperm attains its full size, the outer dividing layer ceases to function, and becomes the young aleurone layer. The cells, which constitute it, retain their embryonic form, as regards both their size and shape, in their adult condition. They eventually thicken their walls considerably, and their contents become packed with aleurone grains, so that finally the nitrogenous layer characteristic of the endosperm of cereals is formed.

Plate XXIV. illustrates a section of the endosperm taken at the stigmatic end of a grain, at a stage when the aleurone layer is not yet adult. The hyphae, which by this time are in the form of a layer, take part in the nourishment of the aleurone cells. Just, as in the case of the starch cells, they actually enter into the cell cavity by penetrating the cell-wall, and become absorbed by the protoplasm which converts the nourishment so obtained into the aleurone grains which are present in great abundance in the adult layer. This plate shows several hyphae passing through the walls and disappearing into the cell-contents.

The aleurone cells figured are young. They show a well-marked nucleus, and are filled with protoplasm, in the meshes of which aleurone grains are being formed. The absorption of the hyphae continues until the cells are packed with grains, and the seed is nearly ready for ripening. A section of a fully mature normal endosperm shows, however, no signs of the endophytic nature of the fungus.

It is interesting to note that Peklo (16) suggested that the aleurone layer was probably fungal in origin in all cereals. The suggestion arose as a result of an incidental examination of some *Lolium temulentum* grains. In order to carry the investigation further he decided to examine grains of *Triticum*, *Secale*, and *Hordeum*. He recognised the necessity of examining rust-resistant types, and stated fully in his paper the varieties he proposed to examine. With the forms chosen he obtained negative results. Not deterred, he next examined material he already had embedded in paraffin, but he did not state its origin, or given any information regarding its rust-resistant capacities.

Examination of such material revealed fungal hyphae occupying the lumen of the aleurone cells, from which densely stained bodies were budded off. Peklo believed them to be aleurone grains. It seems highly probable that the grains used for sectioning were mouldy, and that the aleurone grains figured are in the process of digestion. This is accentuated by the fact that some grains were found actually embedded in the hyphae themselves, and also by the fact that Peklo suggests that the fungal threads found bear a resemblance to those of *Mucor Rouxianus* (*Amylomyces Rouxii*), although the actual identity of the two was not established. The point of interest as far as this paper is concerned lies in the fact that Peklo probably found the fungus in the aleurone cells of young *Lolium temulentum* grains, and from this isolated case he attempted to generalise, stating that such was the origin of the layer for all cereals.

The breadth of the hyphal layer found in the grain is dependent on two factors—

(a) The activity of the fungus.

(b) The absorbing power of the endosperm.

If the fungus is strong and luxuriant in its growth, and can keep pace with the activity of the endosperm, a thick hyphal layer would result, for even at maturity the endosperm will not have used, as food-material for itself, all the available hyphae.

If, however, the growth of the mycelium is inclined to be weak, the absorbing power of the endosperm will be greater than the growing power of the fungus, and the result will be a very small layer in the mature grain, or even perhaps the complete absence of such a layer.

In the earlier part of this paper (p. 256) I emphasised the fact that absence of the fungus in hand-sections, or in any individual microtome section could not be taken as evidence of the total absence of the fungus in the grain. The reason for this statement should now be clear. The presence or absence of a definite layer in the grain is dependent on the activity of the fungus, and the absorbing power of the endosperm. Even if a grain does not exhibit a definite layer, hyphae may still be present in the embryo in sufficient amount to ensure the appearance of the fungus in quantity at the desired stage in the development of the next generation of Lolium.

We are also in a position to discuss the significance of the distribution of the fungus in the grain. Freeman attributed it mainly to the result of the method of infection of the embryo, but I

am led to the conclusion that it is a result of the part played by the fungus during the development of its host. The grass so controls and subjugates the mycelium during the changes which take place after fertilisation, that the embryo-sac, as its increases in size, pushes the fungus closer and closer to the periphery, until the mature condition is reached. Not only is the hyphal layer found between the endosperm and the testa, but, if the fungus is active, remnants may be found all round the periphery of the embryonic area, in fact, in any position occupied by them during the later embryological stages. (Plate XVIII. Figs. 1-5.)

The embryo during this period follows the usual course of development. At Stage C it was an undifferentiated club-shaped body, and hyphae were in close association with its micropylar end.

The next marked period of growth results in the appearance of the stem apex (Plate XXV., Fig. 4.) This is followed by differentiation of the radicle and elongation of the cotyledon. When all the parts of the embryo are thus marked off from one another, growth continues until the embryo is fully developed. The fungus, in the meantime, can generally be seen at both the micropylar end, and also, between the developing scutellum and endosperm. It is generally pronounced in the region of the plerome cells of the cotyledon.

Further investigations of the development of the embryo have been commenced in order to determine more exactly the relation of the fungus to its later development, as it is possible that the fungus plays a role in the formation of the scutellum comparable to the one it plays in the formation of the endosperm.

The hyphae, already in the very young embryo, follow the development of the stem-apex, and remain localised in their growth until germination takes place.

The Fungus in the Plant.

The growth of the fungus keeps pace with that of the plant, the hyphae, however, are mainly restricted to the growing apex, but can be seen extending for a short distance down the stem. They show the dual staining property already described (pg.

Even at this stage the intra-cellular nature of the fungus can be demonstrated. Some of the parenchymatous cells of the grass are invaded, and used as a food supply by the hyphae. Such cells always stain with gentian violet, and they show a dense network of

hyphae. They may occur near the vascular tissue, and also towards the periphery of the stem.

When the inflorescence is formed, they are especially abundant at the base of the carpels. The cells so affected do not increase in size, and are only to be distinguished from a normal unaffected cell by their different staining properties. It is not till the ovule is well advanced that any great increase in the fungal partner takes place, when the phenomena already detailed follow in their natural sequence.

Cultivation of the Fungus in Artificial Media.

All attempts by previous workers to obtain a pure culture of the fungus have been unsuccessful. Their work has been limited mainly to the nucellar hyphae. So far I have been no more successful than Nestler and Freeman in endeavouring to get the fungus to grow outside its host. As further work is being done in this direction, it has been thought advisable to give a short account of the methods employed, and the results so far gained.

Since hyphae isolated from the hyphal layer of the grain had not yielded any result, and as they represent the dormant stage of the fungus, I thought greater success might be attained if the cultures were made from a more active stage in its life-history. Accordingly, the ovary was thought to be a suitable starting point, and stages ranging from A-C in the development of the grass have been used for infecting the culture media.

For the most part the culture medium has been made up in the following way :—

A decoction of *Lolium perenne* in water was autoclaved, then filtered and cleared with egg albumen. The liquid so obtained was made into a 1% agar solution, and autoclaved. It was subsequently filtered, titrated, tubed and sterilised.

Other media have been tried, e.g., honey agar, starch agar, etc., but with no better results.

The ovaries were treated in various ways, before using them for infecting the plates.

(1) Some were washed for one minute in equal parts of a 1% mercuric chloride solution and 45% alcohol, followed by a thorough washing in sterile distilled water.

(2) Others were washed in ether for varying lengths of time, from five minutes to one minute.

(3) Others, again, were shaken for some time in sterile distilled water.

After this preliminary treatment they were crushed with sterile forceps and introduced into the mouth of the agar tube, and then immediately plated. A drop of lactic acid solution being introduced to eliminate the growth of bacteria.

(Crushing the ovaries brings the fungal hyphae into direct contact with the medium used, and it was though that this might induce growth).

Some of the plates were left at room temperature, others were incubated at 23°-25°C. As a rule the plates were found to remain remarkably free from external contamination, and exhibited no growth at all. Occasionally some superficial fungus, mostly *Penicillium*, developed from the surface of the ovary, more especialy from the stigmas.

Probably the preliminary treatment to which the ovaries were subjected may have acted detrimentally on the fungus, even killing it. Further work, however, requires to be done to decide this point.

One plate infected with an ovary, which had previously been immersed in ether for four minutes, exhibited a fungal growth which seemed to arise from the ovary as a centre and which could not be attributed to any of the commoner superficial forms.

The first signs of growth appeared on the third day after infection. The hyphae were extremely septate, and their tips seemed to divide into two, the resulting branches growing equally. At this time there were no signs of spore formation. On the thirteenth day signs of fruiting bodies were noticed. When young they appeared salmon pink in colour, becoming very dark when old. They were irregular in size and shape, and appeared to be of the nature of pycnidia.

I have to thank Mr. C. C. Brittlebank (Government Plant Pathologist) for identifying the growth so obtained. He had no hesitation in placing it as a *Coniothyrium*, probably closely related to *C. olivaceum*, Bon. The ovary from which the felt was obtained was fixed, along with a portion of the felt, in Fleming's weak solution, and afterwards microtomed. The sections showed that the tissues of the ovary remained intact during the growth of the mycelium, and hyphae similar to those composing the felt were found running in its tissues.

This may or may not be the fungus found in the *Loliums*, but its close affinity to *Phoma* is rather suggestive, for many mycorrhizal forms have been found to belong to this latter genus.

The ovary used in this plate was obtained from a plant growing in the Melbourne University grounds. It was apparently the product of a second flowering resulting from heavy early autumn rains. Since all attempts to obtain this form again have been unsuccessful, it might be argued that it cannot be the fungus found associated with *Lolium perenne*. This may be so, but it is just probable that since the ovary represented a second flowering the fungus may be growing more actively than the developing grain, the grass being naturally weakened by its previous flowering, so that the fungus may have been in a suitable condition to grow on the artificial medium provided.

Concerning the Function of the Fungus.

It has been suggested that the fungus associated with Darnel grass possesses the power of nitrogen-fixation. Hiltner (5) was the first to formulate this idea, and after testing it by experiment, he concluded that *Lolium temulentum* grew as well in nitrogen-free sand, as in sand to which nitrogen, in the form of potassium nitrate, had been added as a fertiliser. As a control he grew *Lolium italicum* under similar conditions. This species, at the time of Hiltner's work (1899), was regarded as being fungus free. Later, Freeman (1903) found in a sample of 59 grains two contained the fungus and 57 were devoid of it. This, although it is a low percentage of infected grains, could introduce a serious error into such work when using this species as a control.

The experimental methods employed by Hiltner are also open to criticism. He planted grains of both species in pots, which were completely nitrogen-free, but he watered one set of two with tap-water, which contained 0.84 mg. of nitrogen per litre. To the other set of two he gave in addition 50 mg. of nitrogen in the form of potassium nitrate. These pots were apparently left exposed to the air, and so were subject to many sources of external nitrogen contamination, the most formidable perhaps being nitrogen-fixing bacteria.

An experiment carried out in this manner could not aim at determining whether the fungus is capable of fixing free atmospheric nitrogen in *the compelete absence of combined nitrogen*. However, as several investigators have shown, Berthelot (17), Puriewitsch (18), and Latham (19), that certain fungi can fix free nitrogen if supplied with a small amount of this element in a combined form, the results given by Hiltner might have some bearing on the latter point.

The following figures are extracted from his paper:—

I.—*Without nitrogen manure.*

	Dry Weight gr.	Nitrogen absolute mg.	Nitrogen per cent.
Lolium temulentum	5.173	30.35	0.59
Lolium italicum	0.974	6.69	0.69
Root mixture	3.619	7.78	0.22
Total	9.766gr.	44.82mg	0.46%

II.—*Manured with 50 mg. of nitrogen.*

	Dry Weight gr.	Nitrogen absolute mg.	Nitrogen per cent.
Lolium temulentum	5.867	72.87	1.24
Lolium italicum	2.329	40.70	1.75
Root mixture	3.381	12.60	0.37
Total	11.577gr.	126.17mg.	1.09%

The nitrogen content of *Lolium temulentum* plants, when fertilised with potassium nitrate, is seen by the above figures to increase markedly as compared with that obtained for unfertilised plants, i.e., plants watered with tap water only. Not only is this so, but the increase is nearly as great as that obtained for *Lolium italicum*. The small difference between the percentage results for both species is not outside the limit of experimental error, especially when the sources of such error are as great as in the experiment in question.

Rayner (20) when dealing with the symbiotic relation of an associated fungus in *Calluna vulgaris*, refers to the case of Darnel grass, and says: "Some degree of symbiosis has been inferred, but the experiments of Hiltner to establish nitrogen fixation for this fungus are inconclusive."

In describing the distribution of the fungus (a peculiar mycorrhizal form) found in *Calluna vulgaris*, Rayner draws attention to the fact that in many points it resembles the fungus in Darnel. The fungus from *Calluna* was isolated and grown in pure culture, and was found to be closely related to the genus *Phoma;* nitrogen-fixation was suggested as its function. Duggar and Davis (21) showed that *Phoma Betae*, when grown on mangel or sugar beet decoction, produced a nitrogen gain of from 3.022—7.752 mg., pointing definitely to nitrogen fixation for this particular fungal species. In fact, it was the only definite positive result obtained from all the forms experimented with.

Although many mycorrhizal fungi are thought to aid their host plant in this way, considerable uncertainty exists concerning the determination of the species producing mycorrhiza and their actual function.

These facts suggest that it is not improbable that the fungus associated with Darnel or English rye grass might act as a nitrogen-fixer, so an experiment was devised to try and establish a definite answer to this suggestion as regards *Lolium perenne*.

Materials and Apparatus.

(1) Method of Preparing Sand.

Sand cultures were chosen, as they supply a rather more natural condition for the plant roots than water cultures, and sand has the additional advantage of being practically insoluble, and it does not interact with the nutritive compounds used in the watering solutions. In order to obtain it free from all traces of nitrogen, it was subjected to the treatment recommended by Schramm (22). A good sample of fine quartz sand was chosen. This was thoroughly washed for about two hours in running tap-water. It was next boiled in strong hydrochloric acid for about one hour, and then washed with distilled water until chlorides could no longer be detected on the addition of silver nitrate. The sand was then heated to a red heat in a furnace for eight hours. This effectively removes any organic material which may be present. The ash formed in this way and any remaining traces of nitrogen were removed by a second boiling in pure strong hydrochloric acid. A second washing with distilled water ensued, and was carried on until the sand was free from chlorides. Finally it was washed a dozen times with nitrogen free water, and then dried in a drying oven. After this treatment, on testing for ammonia, nitrites and nitrates, only negative results were obtained.[5]

5. Nessler's reagent was used in testing for ammonia. The Lunge test (Diphenylamine) was used in testing for nitric acid. A modification of the Peter-Griess method was used in testing for nitrous acid. This test is extremely delicate, according to Anderson (25). One-thousandth of a milligram can be detected with certainty. The Griess-Ilosvay method is as follows:—

(1) Dissolve 0.5 gm. of sulphanilic acid in 150 cc. of 2-normal acetic acid.

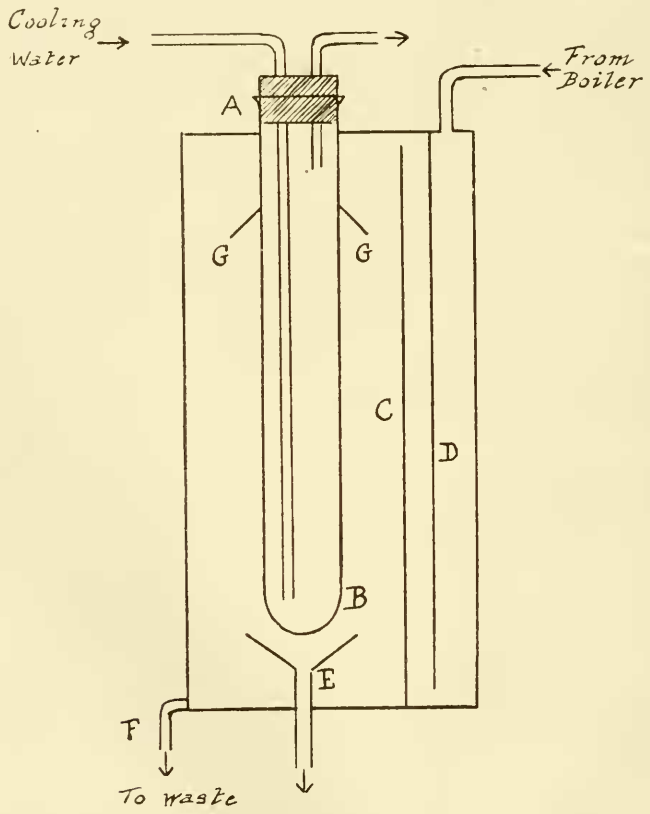
(2) Boil 0.2 gm. of *a* naphythylamine in 20 ccs. of water. Pour off the colourless solution from the violet residue, and add to the solution 150 cc. of 2-normal acetic acid. Mix the two solutions. (This mixture must be kept in a dark place.) Take 50 c.c. of the material to be tested with 2 cc. of above reagent, and allow it to stand 5 or 10 minutes; it will be coloured red if a trace of nitrous acid is present.

In using this test, the flask should be plugged with cotton wool, to prevent dust from entering the solution and disturbing the result.

(2) Low conductivity water.

I have to thank Dr. Rivett, of the Chemistry Department, Melbourne University, for the use of a still, the pattern of which was first described by Hartley, Poole and Campbell (28), and which readily yields water of conductivity as low as 0.4×10^{-6} mhos. The method employed is briefly as follows :—

Ordinary distilled water is boiled for about 10 mins. in a 10 litre copper vessel, open to the air, and this is connected to the apparatus shown in text-figure 8.



Text-Figure 8.

A diagram of the condenser used in preparing low condctivity water, AB = condensing tube; E = collecting funnel; C and D = Copper sheet baffles; G = tin flap welded to AB to prevent condensate from soldered junction running down A.B.

The essential part is the condensing tubes A B, which is of pure tin. Before the entering steam can reach the condenser, it must travel round two copper-sheet baffles C and D, which effectively retain liquid particles projected from the boiler. The condensate drips into the tin-funnel E, and is collected in a 3-litre Jena glass flask, with the usual guard tubes of soda lime. It is removed from the flask by siphoning. The outer cylinder is of copper, and is protected by asbestos sheeting on the outside. No water which condenses on the cylinder or the copper baffles-plates can enter the funnel E; it is drained away at F. To prevent the condensate running down A B from the soldered junction of the tube with the top of the cylinder, a tin flap is welded on, as shown at G. The drippings from this fall outside the funnel E. The middle fraction of 3 litres is the purest.

Apparatus.

Two large glass shades, fitted into a groove round the periphery of wooden stands, were used for covering the pots, in which the grains were planted. These were carefully cleaned with acid-dichromate cleaning mixture. In each case a long glass tube bent at right angles to itself was inserted through the stand into the cylinder. These were connected in turn to a series of wash-bottles. The first of these contained chemically pure sulphuric acid, giving no nitrogen reactions. This acid bottle was connected to two series of water-wash bottles, one set belonging to each cylinder. The connections were made with glass and rubber tubing. Between the last wash-bottle and each shade, a tube of wider diameter was inserted, containing a germ-proof cotton plug. A second tube of smaller length, also bent at right angles and fitted through the wooden base into the other side of the cylinder functioned as an exit tube. These in turn were connected each to a wash-bottle, and then to a water pump. By this means a slow current of air could be kept drawn through the cylinders. Before reaching them, the air had to pass through the acid, two wash-bottles containing nitrogen-free water, and finally through the cotton plug, so all combined nitrogen in the form of dust, etc., was removed before the air reached the pots. This treatment also reduced to a minimum the chance of nitrogen fixing bacteria, present in the atmosphere, gaining access to the sand.

After the experiment had been running for some little time, it was evident that a large amount of water was being carried over

by the air and condensing on the sides of the glass cylinders. In order to prevent the air from being too moist, U-tubes containing pure calcium chloride were inserted betweeen the last wash-bottle and the cotton plug.

Watering with the nutritive solutions was carried out by a siphon arrangement. A glass tube bent at an angle over each pot was connected by rubber tubing to a separating flask. This was tightly stoppered, and the stopper covered by a small inverted beaker to prevent dust from falling on it. By raising or lowering this flash the solutions flowed freely on to the pots and the quantities given could be altered at will.

The pots themselves had glazed surfaces, and were quite nitrogen free.

Before setting up the experiment, all the glass bottles and tubing were washed with the cleaning mixture, and then several times with nitrogen-free water. The open ends were plugged with cotton wool and sterilised, in a steam steriliser, on three successive days. The rubber stoppers and tubing were boiled in dilute alkali, then in dilute acid, and subsequently washed with nitrogen free water. Connections were made as soon as possible after removing the plugs.

The slot in the stand into which the cylinders fitted was sealed with putty, all the other joints were sealed with paraffin. There was every indication that the connections were air-tight.

The grains before planting were treated with a 2% formalin solution for 8 minutes, then washed thoroughly in nitrogen-free water. By previous trial it was found that this treatment did not affect the germination capacity of the seeds, and rendered them as sterile as possible.

The sand, being prepared in the manner already described, was sterilised, left to cool, moistened with nitrogen-free distilled water, and the grains planted. The shades were immediately fitted into place, the connections made, and the experiment commenced running on August 18th, 1919. It was so arranged that an equal amount of illumination was received by both pots.

The drying tubes soon became saturated with water, and it was found necessary to change them every second day. The U-tubes when not in use were kept sterilised and plugged, so that the sterility of the system was not affected by this factor. The sulphuric acid and water in the wash bottles was also changed occasionally, so as to prevent any traces of nitrogen accumulating in

the last wash bottles, and thereby reaching the cylinders. During the process of changing the bottles, the rubber connections with the cylinders were clamped, so that air could not reach them.

(4) *Nutritive solutions.*

The control pot was watered with a nutritive solution, made up according to the following formula :—

Ammonium nitrate, 0.5 gram.
Potassium di-hydrogen phosphate, 0.2 gram.
Calcium sulphate, 0.1 gram.
Magnesium sulphate, 0.1 gram.
Sodium chloride, 0.1 gram.
Ferric chloride, 0.04 gram.
Nitrogen-free water, 1000 ccs.

The second pot was watered with a similar solution, excluding the ammonium nitrate.

The chemicals used were the purest that could be obtained. The watering solutions when ready for use gave negative results, with the nitrogen tests already described.

Results.

August 18th.—Experiment commenced.

August 27th.—Grains were germinating freely in both pots.

September 15th.—The seedlings in the control pot were taller and were showing a better colour than those deprived of nitrogen.

September 16th.—First signs of yellowing at tips of leaf in nitrogen-free seedlings.

September 19th.—All the seedlings in the nitrogen-free cylinder showed their first leaf distinctly yellow at tip, and the yellow colour was extending back along the edges of the lamina. The seedlings were all in the two leaf stage. The second leaf was quite green. The seedlings in the control cylinder looked very healthy. No signs of discolouration were evident in them.

September 30th.—The nitrogen-free seedlings were about one-third the height of the control seedlings. The first leaf was very much discoloured and withered. The second leaf was still green, showing no signs of yellowing. The third leaf just visible. The control seedlings were healthy, and of a good green colour; they showed 5-6 leaves.

October 13th.—The nitrogen-free seedlings were very unhealthy. The second leaves showed discolouration, and were dying from the tip downwards. Very much behind the control.

October 20th.—The nitrogen-free seedlings were beginning to die out. The control seedlings were exceedingly vigorous and normal.

October 30th.—The experiment was dismantled for photographic purposes, as the remaining nitrogen-free seedlings were failing rapidly.

The phenomena noted during the course of the experiment are typically those resulting from nitrogen starvation. The yellowing of the older leaves always commencing at the tip indicates that these members are being sacrificed in order that any nitrogen they possess (i.e., nitrogen obtained from the seed) may be made available for transference to the young developing leaves. This transference of nitrogen from the first-formed leaves to the actively growing centre enables the plant to exist for a certain period of time, but the lack of nitrogen manifests itself in the stunted growth and unhealthy colour and appearance of the plants.

It may be concluded from this experiment that no power of nitrogen-fixation in the absence of external supplies of combined nitrogen can be ascribed to the endophytic fungus of *Lolium perenne*.

Conclusion.

It is difficult to decide what is the actual relationship between the fungus and the Lolium plant. It could, perhaps, be regarded as a case of symbiosis, the fungus helping in the plant economy during the formation of the grain. In return for this it is housed by the grass, and its propagation is ensured by the admittance of hyphae to the embryo, so that it is able to appear in each successive generation without the intervention of a spore stage. It can only be a matter of conjecture whether this stage is entirely lost to the fungus or whether it is repressed, only as long as conditions are favourable to its transmission in the usual way, but still retains the power of sporing if in danger of extermination. The sporing stage may occur under such conditions, but up to the present it has not been recognised as belonging to the fungus normally found associated with at least two species of Lolium.

As opposed to this conjecture, Freeman (26), although he had not demonstrated the intra-cellular nature of the fungus, advocated

the view that it was a smut. He was drawn to this conclusion by the idea "of the probable progression of the evolution of parasitism in smuts," from the loose smut of oats through the loose smut of wheat to the Lolium fungus. The loose smut of wheat, forming as it were, an intermediate stage between the loose smut of oat and the fungus of Lolium. It is well known that the spores of the loose smut of wheat infect the ovary at the flowering stage, and there form a mycelium, which perennates in the embryo of the grain, growing when it germinates in the manner of most smuts, and forming its spores during the next flowering stage.

The Lolium fungus could be regarded as a development from a type such as this, in which the sporing stage has been entirely suppressed, or at most occurs extremely rarely.

The points in the life-history of the fungus associated with *Lolium perenne*, which could be used to support this view, are:—

1. The behaviour of the hyphae during the growth of the plant up to the flowering stage, which closely resembles the method adopted by the *Ustilagineae*.
2. The formation of knots in the tissue of the young ovary, which, however, I prefer to explain, not as an attempt towards spore-formation, but as the preliminary to cell infection.
3. The intra-cellular course of the hyphae.

These, at first sight, undoubtedly seem to weigh heavily as evidence in favour of its relation to the smut family, the most suggestive being the behaviour of the fungus during the vegetative period of the grass.

This mode of growth in the host plant, however, is not limited to the *Ustilagineae*. Rayner (20) describes a fungus associated with *Calluna vulgaris* which grows in the tissues of the plant without any external evidence of its presence or without disturbing the normal growth of the host.

The greatest development of the fungus in this case takes place on the roots of the plants forming a mycorrhiza, endotrophic in character, but unlike most other mycorrhizal plants, the fungus keeps pace with the growing point of the stem, and after the inception of the ovary it enters this organ, and forms a mycelium in the ovary wall. The embryo remains sterile, but infection of the seed-coat is accomplished by the hyphae, so that the production of a mycorrhiza in the roots of the next generation of Calluna is not left to chance.

The fungus in the seed-coat becomes active on germination, infects the seedling, and produces the felt on the root; indeed the association is so close that the symbiosis is an obligative one, the seedlings not developing unless infected.

This is a very striking instance of symbiotic association, and, as Rayner points out, the only other plant for which a like distribution of the fungus has been described is *Lolium temulentum*. Since I have demonstrated the intra-cellular nature of the Lolium fungus it falls even more in line with the Calluna type. Although it does not form a mycorrhiza, its use to the plant is certainly demonstrated at the fruiting period. It is possible, therefore, to regard it as a case parallel to the Calluna type, the similarity to the Ustilagine mode of growth being accidental, and not of any real importance in helping us to classify it and to grasp its affinities. Since the Calluna fungus has yielded to artificial culture, and can be classed definitely as a *Phoma*, every hope can be entertained for success in this direction as regards the fungus of the Loliums.

The occasional penetrations of the endosperm, etc., already described, do not, I think, point to vestigial traces of a former parasitic habit. Even when present they do not evince any harmful results in the grain. They are probably to be explained as a luxuriant development of the fungus, resulting perhaps from good growth conditions. The endosperm has proved unable to cope with the large food supply represented by the fungus, and consequently has failed to transform it all into the usual storage form—starch and aleurone grains, so that some of the hyphae which had penetrated the tissues of the embryo, and would normally have been absorbed, remained intact. Any food-material they contained would be yielded up on germination, just as the food-material of the endosperm is changed into soluble forms, and translocated to the seat of growth.

Although I have never examined a grain of *Lolium perenne* either mature or in an embryonic condition without finding the fungus present (sometimes in minute amounts), it is quite probable that such occur.

It is conceivable that the hyphae, when growing in the young inflorescence may miss a carpel and in that case the ovule would not become infected. This would probably not prevent the formation of a fruit. The grain so formed, however, would not be so well equipped in its struggle for existence as its fungal-containing neighbour, and eventually would tend to die out. It is, therefore,

unlikely that two races of both *Lolium temulentum* and *Lolium perenne* exist, one with a symbiont, the other fungus free. An occasional grain of either species may show the absence of hyphae, but this would be accidental in character, so that instead of an ever-increasing number of the latter type, they would always tend to remain at a more or less stationary minimum.

Summary.

The foregoing investigation has led to the following results :—

(1) The occurrence of the fungus in the genus *Lolium* is wider and more constant than has hitherto been demonstrated.

(2) Colour of the grain cannot be regarded as a diagnostic character in regard to the presence or absence of the fungus.

(3) The fungus is intra-cellular or endophytic in nature.

(4) The distribution in the grain is not a result of any special method of infection, but is a result of the function of the fungus during the grain's development.

(5) It is present in the embryo-sac at or immediately after fertilisation.

(6) The fungus increases in quantity at the expense of the nucellus, and the cells of the carpel wall. This is only a temporary phase. On the formation of endosperm the fungus is absorbed as a source of food-supply to the developing embryo.

(7) The endosperm is formed by the division of its outer layer. This layer functions as a kind of cambium. I have termed it the *endospermic cambium*. The cells which are cut off always to the inner side, increase in size, remain thin-walled, and become packed with starch. This outer meristematic layer is constantly receiving and absorbing hyphae, which, if present in any quantity, are finally crushed into a layer around the periphery of the endosperm. If the fungus does not keep pace with the absorbing power of the endosperm, no hyphal layer is formed in the ripe grain, but hyphae can then be found in the scutellum and embryo.

(8) The endospermic cambium after it has ceased to divide persists as the aleurone layer, which, in turn, receives a supply of nutriment from the fungal system.

(9) The ovum is infected before any divisions have taken place in it.

(10) The hyphae aggregate at the proximal end of the developing grain. They are here used by the endospermic cells in the embryonic pocket for food-supply for the developing embryo.

(11) The association of the fungus with *Lolium temulentum* and *Lolium perenne* is probably a well-marked case of symbiosis, comparable in many respects with that met with in *Calluna vulgaris*.

(12) It has been suggested that nitrogen fixation was the function of the fungus, but an experiment has been performed, and the result obtained showed that the fungus of *Lolium perenne* is unable to fix nitrogen in the total absence of external supplies of combined nitrogen.

The foregoing work was carried out in the Botanical Department of the Melbourne University during the years 1917, 1918, and 1919. I have to thank Professor Seward, Cambridge; Mr. S. F. Armstrong, Agricultural School, Cambridge; Dr. Stoward, Western Australia; Mr. Burtt Davey, South Africa; Mr. Breakwell, Sydney; Vilmorin-Andrieux and Cie, Paris; and the Royal Botanic Gardens, Kew, for forwarding supplies of grain from different parts of the world; also Mr. O'Brien, Assistant in the Botanical Department, Melbourne University, for the help he has afforded in assisting with experiments and taking photographs. To Professor Ewart I am indebted for the facilities provided for the work, and for much helpful criticism during its progress.

BIBLIOGRAPHY.

(1) Vogl, A. E.—Zeitschrift für Nahrungsmitteluntersuchung, Hygiene und Waarenkunde, Vol. XII., Jan., 1898.

(2) Guérin, P.—"Sur la présence d'un Champignon dans l'Ivraie."—Journ. de Bot., Vol. XII., Aug. 1898.

(3) Hanausek, T. F.—"Vorläufige Mitteilung über den von A. Vogel in der Frucht von Lolium temulentum entdeckten Pilz."—Ber der Deut. Bot. Ges., Vol. XVI. 1898.

(4) Nestler, A.—"Über einen in der Frucht von Lolium temulentum, Lolch entdeckten Pilz."—Ber. der Deut. Bot. Ges., Vol. XVI. 1898.

(5) Hiltner, L.—"Uber die Assimilation des freien atmosphärischen Stickstoffs durch in oberirdischen, Pflanzenteilen lebende Mycelien."—Centralbl. f. Bakt. u. Parasiten, Vol. V., 1899.

(6) Micheletti, L.—"Sulla tossicita dei semi di Lolium temulentum, L."—Bull. d. Soc. Ital., Vol. VI., 1901.

(7) Freeman, E. M.—"The seed-fungus of Lolium temulentum, L., the Darnel."—Phil. Trans. of the Roy. Soc. of London, Series B., Vol. 196, 1903.

(8) Nestler, A.—"Zur Kenntnis der Symbiose eines Pilzes mit dem Taumellolch."—Sitzungsberichte der Kaiserlichen Akademie der wissenschaften, Vol. CXIII., 1904.

(9) Fuchs, J.—"Beitrag zur Kenntnis des Lolium-pilzes."—Hedwigia 1911.

(10) Buchet, Samuel.—"Le cas du Lolium temulentum et celui de l'Althaea rosea."—Paris Bul. Soc., Bot. 59, 1912.

(11) Brown, H. T., and Morris, G. H.—"Researches on the Germination of some of the Gramineae."—Journ. of the Chem. Soc., Vol. LVII., 1890.

(12) Brown, H. T., and Escombe, F.—"On the Depletion of the Endosperm of *Hordeum vulgare* during Germination."—Proc. of the Royal Soc. of London, Vol. LXIII., 1918.

(13) Coulter and Chamberlain.—"Morphology of Angiosperms."—University of Chicago Press, Chicago.

(14) Cannon, W. A.—"A morphological Study of the Flowers and Embryo of the Wild Oat, *Avena fatua*, L."—Proc. of the California Acad. of Sci., Third Series, Vol. I., No. 10, 1900.

(15) Brenchley, Winifred.—"The Development of the Grain of Barley."—Annals of Botany, 1912.

(16) Peklo, Jaroslav.—"Uber die Zusammenseizung der sogenannten Aleuronschicht."—Ber. d. Deut. Bot. Ges., Bd., XXXI., 1913.

(17) Berthelot, M.—"Recherches nouvelles sur les micro-organismes fixateurs de l'azote."—Compte-Rend, Acad. Sci., Paris, 116, 1893.

(18) Puriewitsch, K.—"Uber die Stickstoffassimilation bei den Schimmelpilzen."—Ber. der Deut. Bot. Ges. Vol. XIII., 1895.

(19) Latham, Marion E.—"Nitrogen assimilation of Sterigmatocystis nigra and the effect of Chemical Stimulation."—Bull. Torr. Bot. Club, 36, 1909.

(20) Rayner, M. C.—"Obligate Symbiosis in Calluna vulgaris."—Annals of Botany, Vol. XXIX., 1915.

(21) Duggar, B. M., and Davis, A. R.—"Studies in the Physiology of the Fungi—I. Nitrogen Fixation."—Annals of the Missouri Botanical Gardens, Vol. III., 1916.

(22) Schramm, J. R.—"A Contribution to Our Knowledge of the Relation of Certain Species of Grass Green Algae to Elementary Nitrogen."—Annals of the Missouri Botanical Gardens, Vol. I., 1914.

(23) Koch, A.—"Handb. tech. Myk.," by Lafar, Vol. III.

(24) Goddard, H. N.—"Can Fungi Living in Agricultural Soil Assimilate Free Nitrogen?"—Botanical Gazette, Vol. LVI., 1913.

(25) Anderson, V. G.—"The Determination of Nitrites in Water."—Soc. of Chemical Ind. of Victoria, Vol. XIII., 1913.

(26) Freeman, E. M.—"The Affinities of the Fungus of Lolium temulentum, L."—Annales Mycologici, Vol. IV., 1906.

(27) Freeman, E. M.—"Symbiosis in the genus Lolium."—Minnesota Botanical Studies, 1904.

(28) Hartley, H., Campbell, N. H., and Poole, R. H.—"The Preparation of Conductivity Water."—Journ. of Chem. Soc., Trans., 1908.

EXPLANATION OF PLATES.

All figures have been drawn with the aid of the camera lucida (Zeiss).

Plate XVIII.

Figures 1-5 stained with aniline gentian violet, followed by Gram's iodine wash. They illustrate the distribution of the fungus in the grain, the position of the hyphae is indicated by the yellow line shown in the figures.

Fig. 1.—A semi-diagrammatic representation of a transverse section through the distal part of a grain of *Lolium perenne*.

(a) aleurone layer; (b) cells of aleurone layer, which stain differently from the rest, probably ferment-containing cells; (e) starchy endosperm; (h) hyphal layer; (h^1) coloured portions of hyphae (the great bulk of the layer is not stained with the gentian violet); (t) pericarp and testa.

Fig. 2.—A transverse section of the proximal end of the same grain. Letters as in Fig. 1, and (s) scutellum; (el) epithelial layer of scutellum.

Fig. 3.—A transverse section of the extreme proximal end of the grain. Letters as before, and (r) radicle; (ht) hyphae in fused pericarp and testa.

Fig. 4.—A longitudinal section of a grain of *Lolium perenne* taken in the coronal plane. Letters as in Figs. 1-3, and (g) growing point of embryo; (l) sheathing leaf.

Fig. 5.—A longitudinal section of a grain of *Lolium perenne* taken in the sagittal plane. (m) embryo; (v) vascular bundle of scutellum; (i) ligule; (ht) hyphae in fused pericarp and testa.

Plate XIX.

The following figures have been drawn from sections of a grain of *Lolium perenne* (South Africa). The fungus is especially luxuriant, its intra-cellular nature being evident in the mature grain.

Fig. 1.—A sagittal longitudinal section of a grain of *Lolium perenne*, not passing through the median line. The scutellum shows numerous hyphae, which have gained entrance to this tissue from any point on its surface.

(e) starchy endosperm; (a) aleurone layer; (el) epithelial layer; (h) hyphae in scutellum; (h^1) hyphae round periphery of the scutellum; (c) cells invaded by the hyphae. × 250 diam.

Fig. 2.—Detail of the scutellum.

(h) hyphae passing through the scutellar cells; (c) constriction of hypha during penetration of cell wall. × 1700 diam.

Fig. 3.—Aleurone cells, showing the intra-cellular course of the hyphae,

(a) aleurone cells; (w) wall of aleurone cell; (h) hypha passing from one cell to the next; (h^1) hypha lying at a different level, but drawn in the same plane in figure. × 1700 diam.

Fig. 4.—Detail of scutellum, showing the cells invaded by hyphae. × 1100 diam.

Fig. 5.—Wall of aleurone cell, showing hypha entering into cell through pit in its wall

(p) pit in wall; (h) hypha. × 1100 diam.

Plate XX.

A longitudinal section of an ovary of *Lolium perenne*, just prior to fertilisation; stained with bismarck brown, then with gentian violet, followed by Gram's wash.

(c) carpel wall; (s) starch groups in cells of carpel wall; (i) invaded cells at stalk end of section; (h) hyphae in stalk of ovary; (h^1) hyphae in distal part of wall; (h″) hyphae in distal part of wall not staining with gentian violet; (t) cells in stigmatic tract being used by the fungus as food; (ov) ovule; (hy) hyphae distributed in all parts of ovule; (ic) infected cells of ovule; (es) embryo-sac; (o) ovum; (hy^1) hyphae in embryo sac. × 103 diam.

Plate XXI.

Figures 1-6 show detail of ovule illustrated in Plate XX. (Stage A).

Fig. 1.—Three cells of nucellus, showing the intra-cellular nature of the fungus. These cells react to the violet stain, the rest of the nucellus staining with bismarck brown.

(n) nucleus; (h) hyphae; (h^1) fine ramifications of hyphae in the cells. × 1100 diam.

Fig. 2.—A cell from the carpel wall which is being attacked by the fungus and used as food-material for the fungal system.

(h) hyphae running between the cells; (h^1) fine penetrating threads; (g) starch grains being digested by hyphae; (s) septum in hyphae. × 1100 diam.

Fig. 3.—"Knots" formed by the hyphae wrapping round the cells as a preliminary to their entrance into them. The lightly-shaded portions are lying at a lower level than the darker sections of the hyphae. × 1100 diam.

Fig. 4.—A vascular element from the stalk end of the carpel wall, showing the close association of the fungus.

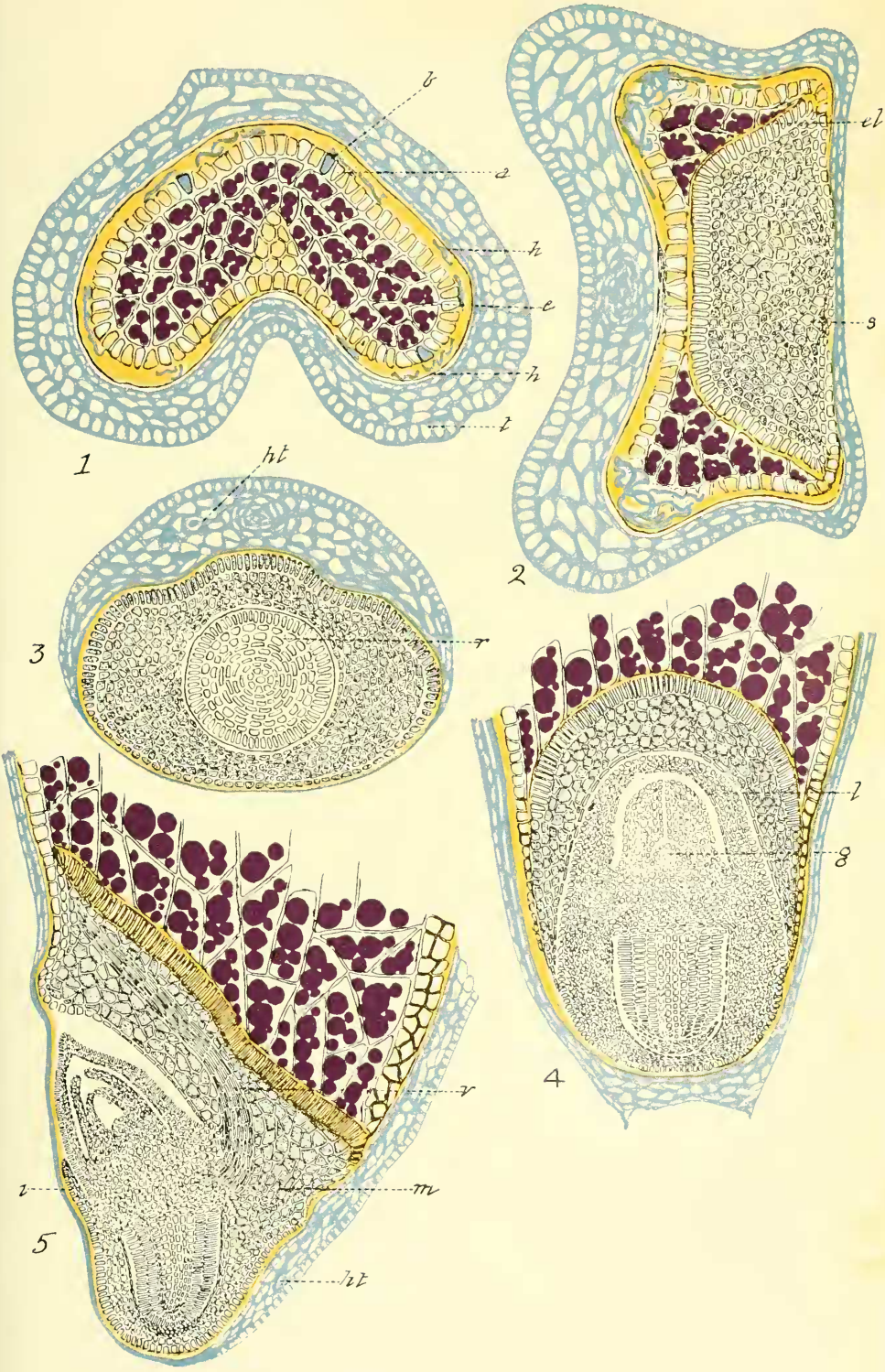
(r) thickenings on vessel; (h) hyphae. × 1100 diam.

Fig. 5.—Cells from the distal end of the carpel wall.

(g) starch groups; (h) large intra-cellular hyphae; (h^1) fine hyphal threads completely wrapping round grains prior to digesting them. × 1100 diam.

Fig. 6.—Ovum and synergidae; showing presence of hyphae in ovum before any divisions have occurred in it.

(o) ovum; (s) synergidae; (n) nucleus (nuc) nucellus; (h) hyphae. × 700 diam.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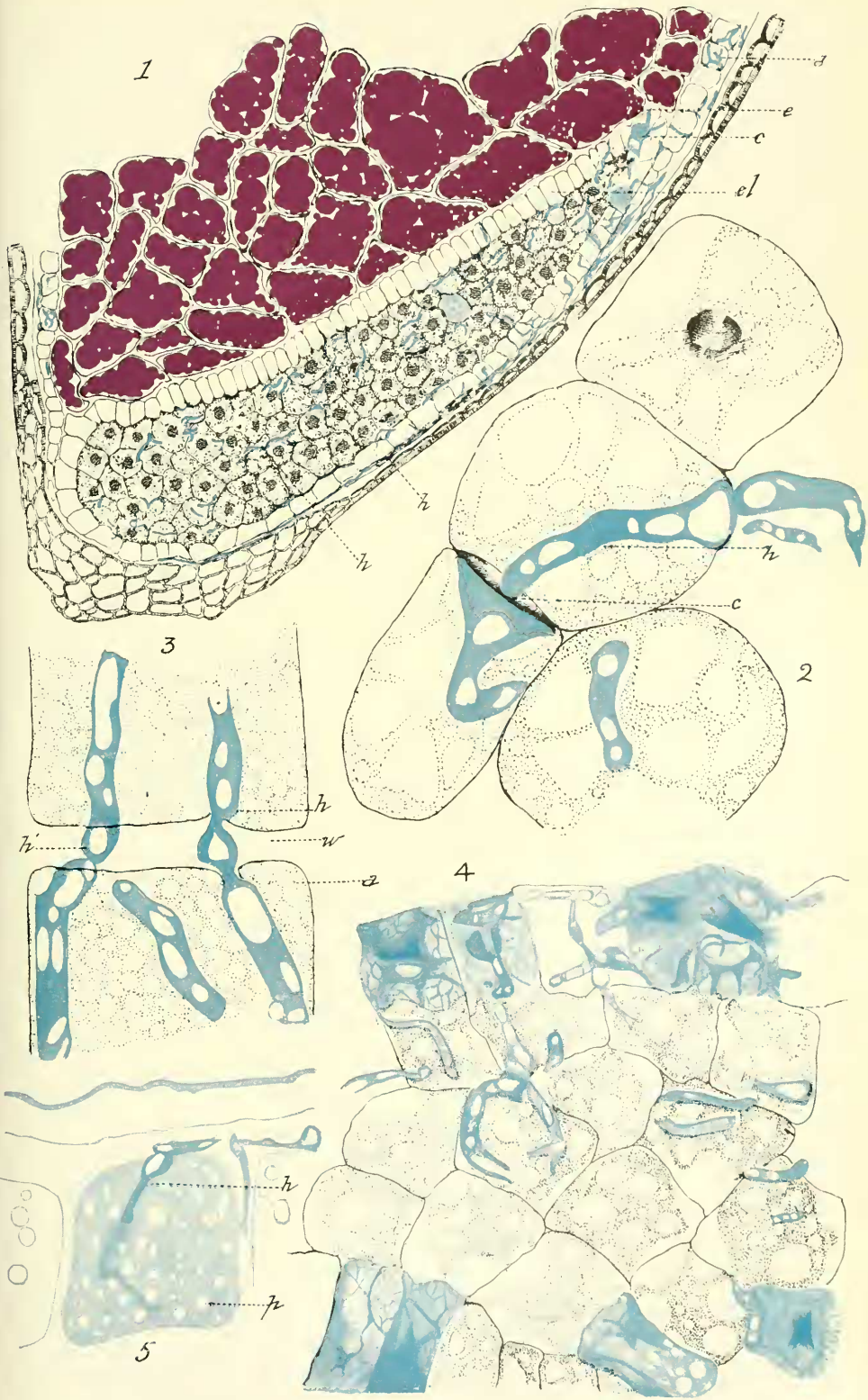
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